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A RAPID DOT IMMUNOASSAY FOR THE DETECTION OF SERUM ANTIBODIES TO EASTERN EQUINE ENCEPHALOMYELITIS AND ST. LOUIS ENCEPHALITIS VIRUSES IN SENTINEL CHICKENS

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Abstract. A dot enzyme-linked immunosorbent assay utilizing a novel membrane, polyvinylidene difluoride, is described. This assay was developed for the rapid detection of serum antibodies to eastern equine encephalomyelitis virus and St. Louis encephalitis virus in sentinel chickens. Antigens were spot-filtered through the membrane. Membranes were dipped into small vials of sera. Antigen-antibody complexes were detected with enzyme-conjugated antiglobulin which, when exposed to substrate, produced a colored insoluble product. The antibody detection protocol was completed within 50 min and was compared with a standard plate enzyme immunoassay. Chickens were experimentally infected with eastern equine encephalomyelitis and St. Louis encephalitis and bled on a daily basis. The dot immunoassay correctly identified 99% (123/124) of the eastern equine encephalomyelitis virus and 100% (67/67) of the St. Louis encephalitis virus antisera. Sera from sentinel chicken flocks in Maryland were also assayed. These data indicate that the dot immunoassay should be considered as an alternative to current assays for the screening of sera for antibodies to virus antigens. This assay could easily be performed in the field and allows for the screening of antibodies to several different viruses in one test.

Enzyme immunoassays (EIA) are becoming common in rapid diagnostics for both antigen and antibody detection.[1] Successful tests have been developed for several arboviruses including eastern equine encephalomyelitis (EEE), St. Louis Encephalitis (SLE), and Highland's J (HJ) viruses.[2–4] Sensitivity and specificity of EIA have been shown to be as good as fluorescent antibody (FA),[2] radioimmunoassay (RIA),[5] and hemagglutination inhibition (HI) tests.[6]

EEE and SLE viruses are significant public health threats.[7,8] EEE virus infects humans and horses, frequently causing acute disease with a high rate of mortality.[9] SLE virus is a major cause of viral encephalitis in humans.[8] One means of combatting epidemics of these diseases is to identify a given area of high risk and subsequently control the vectors. Assaying sera from sentinel flocks of chickens has been shown to be an effective and sensitive method for arbovirus surveillance.[6] EIA is a promising alternative to traditional assays for screening of sentinel animal sera.[6] The technique does, however, require trained personnel to perform and read the test and specific equipment, and may take several hours to complete, therefore, it is not generally adaptable to field use.

We describe here a rapid dot immunoassay (DotIA) for the detection of antibodies to EEE virus and SLE virus in chicken sera. This assay uses a novel membrane, polyvinylydine difluoride (PVDF), and yields a colored, insoluble product within 50 min. The assay requires no special equipment and may be easily performed in the field.

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MATERIALS AND METHODS

Viral antigen preparation

Inactivated EEE and SLE antigens were obtained from the Reference Center for Arthropod-Borne Viruses, Yale University, New Haven, Connecticut. This material had been prepared as sucrose-acetone extracted virus-infected suck-

ling mouse brain.[10] Lyophilized material (0.25 g) was reconstituted in 1.0 ml phosphate buffered saline (PBS), pH 7.4.

Sera

Chickens were obtained from Truslow Farms, Chestertown, Maryland. At 6 days of age, chicks were divided into four groups of six birds. One group was inoculated with 0.9 ml diluent (cell culture medium with 20% heat-inactivated FCS) as a control. Birds in the remaining three groups were inoculated intramuscularly with $10^{3.8}$ $TCID_{50}$ EEE virus, $10^{3.3}$ $TCID_{50}$ SLE virus, or $10^{3.8}$ $TCID_{50}$ HJ virus. Virus strains, bleeding procedures, and processing of blood are the same as described by Scott and Olson.[3]

Sentinel flocks of chickens were maintained in Prince Georges, Ann Arundel, Baltimore, Wicomico, and Worcester counties, Maryland. These birds were bled once a week or every other week. Blood was collected and processed as described above.

Enzyme immunoassays

A microtiter plate based EIA was performed on all samples of sera along with the DotIA assays. The standard EIA was essentially as described elsewhere.[6] Briefly, IgG assays were performed by coating Nunc-Immuno plates (Nunc, Denmark) with polyclonal mouse ascites antibody[2] to the virus of interest. Plates were then incubated with viral antigen for 1 hr at 37°C. After washing, serial two-fold dilutions of chicken sera beginning at 1:100 were added and incubation continued at 37°C. Plates were washed and pretitrated horseradish peroxidase-conjugated goat anti-chicken IgG (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) added and incubated at 37°C for 1 hr. Substrate used was 2.2'-azino-di-[3-ethyl-benzthiazoline sulfonate] (ABTS).

Plates were optically scanned on a Titertek multiscan (Flow Laboratories, Alexandria, Virginia) reader. Controls included sera from non-immunized chickens and normal mouse brain as a negative antigen. Positivity was defined as color development equal to or greater than the mean + 3 standard deviations of the difference between the positive antigen signal and negative antigen signal of negative control sera. EIA titer values are expressed here as reciprocals of the highest dilution of serum that gave a positive test result.

Dot immunoassay

All steps were performed at room temperature (22–25°C). Optimal serum, antigen, and conjugate dilutions were determined by box titrations. Serum controls were normal chicken sera and antigen controls were sucrose-acetone dried uninfected mouse brain at dilutions equivalent to those of the material tested.

Assays were done on a hydrophobic PVDF membrane (Immobilon Transfer, Millipore Corp., Bedford, Massachusetts). Virus infected suckling mouse brain preparations were diluted 1:25 in PBS (pH 7.4) and then spotted onto a PVDF membrane. Spotting was achieved by placing a 1 ml tuberculin syringe onto the membrane and injecting 50 μl of the antigen preparation through it. The membrane was then allowed to dry at room temperature for at least 1 hr. The hydrophobic membrane was wetted in a solution of PBS with 0.2% Tween 20 and then nonspecific binding sites were blocked by incubating the membrane in a solution of 5% nonfat dry milk in distilled water for 15 min. Prepared membranes were then dried and stored at 4°C for later use. Membranes must be rewetted before use in the assay by immersing them in the wash solution of PBS/0.05% Tween 20, prior to initiating the assay.

Antibody assays were performed by dipping PVDF strips with antigen dots into Eppendorf microfuge tubes containing 50 μl of serum diluted 1:10 with PBS containing 0.05% Tween 20 and 2% bovine serum albumin (BSA). Incubation was for 15 min. PVDF strips were then washed by placing them in a container with 50–100 ml of wash solution (PBS/0.05% Tween 20) and gently agitated for 5 min. After washing, PVDF strips were incubated with peroxidase-conjugated anti-chicken IgG for 15 min. PVDF strips were washed as before and placed in a final wash of distilled water for 1 min. PVDF strips were then placed in a substrate solution of 4-chloro-1-naphthol and color development allowed to occur for approximately 10 min. Samples were scored qualitatively by eye from no reaction (0) to maximal color development (+4).

RESULTS

All EEE virus-inoculated chicken serum samples were positive by EIA and DotIA by day 4. Very high (6,400–12,800) antibody titers were observed by day 8; maximum titers were reached

TABLE 1
Comparison of DotIA/EIA values for experimentally infected birds

ELISA titer	EEE DotIA value					SLE DotIA value					Composite* DotIA value	
	0	1	2	3	4	0	1	2	3	4	EEE	SLE
0	18	1				35					0	0
100		1					1	1			1	1.5
200		2	2				1	4			1.5	1.8
400		1	4					2			1.8	2
800			3					1	2		2	2.6
1,600			2	5					4		2.7	3
3,200			1	10	7				3	2	3.3	3.4
6,400				2	18			1	5	5	3.9	3.4
12,800				1	46						4	
Total No. tested					124					67		

* Based on multiplying the number of samples by the correlating DotIA value and calculating the average of the total.

by the tenth day after infection. These values remained constant to day 30. In contrast, only 3 of 6 SLE virus-inoculated birds developed antibody detectable by EIA or neutralization assay. In the 3 birds that did produce antibody, titers were first detectable on days 10–15, rising to peak values on approximately day 20.

DotIA results were compared with EIA data (Table 1). All EEE samples judged as positive by EIA were also detected by DotIA. A EIA titer of ≥100 was reactive in the DotIA assay. The DotIA correctly identified 99% (123/124) of EEE and 100% (67/67) of SLE sera. Correlation of DotIA with low EIA reciprocal serum dilution titers of 100–200 was 100% (4/4) for EEE and 100% (10/10) for SLE. Eighteen of 19 (95%) EEE and 35/35 (100%) SLE samples negative by EIA were also negative by DotIA.

A comparison was made between DotIA value and EIA titer for each sample (Figs. 1, 2). A logarithmic correlation was calculated for averaged values between the two tests (Fig. 3). Variability in EIA titer was observed with samples having a DotIA value of +1 and +2. However, samples having a DotIA value of +3 or +4 were more normally distributed. A strong color reaction (+3) developed at an EIA titer of ≥800. This occurred on days 5 or 6 (2–3 days after antibody was first detected) for the majority of samples from birds experimentally infected with EEE virus. Color reaction for similarly titered SLE samples was the same.

The only sentinel chickens that seroconverted to EEE or SLE viruses were located in Worcester County (Pocomoke cyprus swamp). Antibody to EEE virus was detected in 3 of 4 birds at that location. Four serum samples from each of 3 EEE-positive chickens were tested by EIA and DotIA. Four of the 12 samples were negative by EIA and DotIA. Of the remaining 8 sera, 3 titered

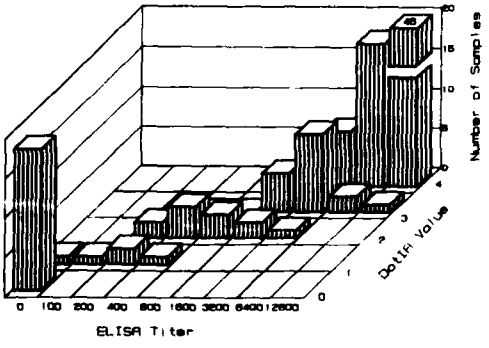

FIGURE 1. Comparison of ELISA titer with DotIA titer by number of correlating serum samples in EEE-inoculated birds. Number of samples scale is truncated at 20.

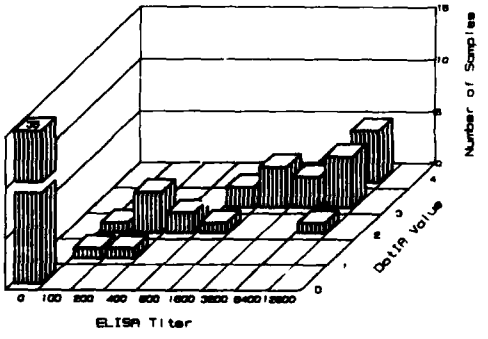

FIGURE 2. Comparison of ELISA titer with DotIA titer by number of correlating serum samples in SLE-inoculated birds. Number of samples scale is truncated at 15.

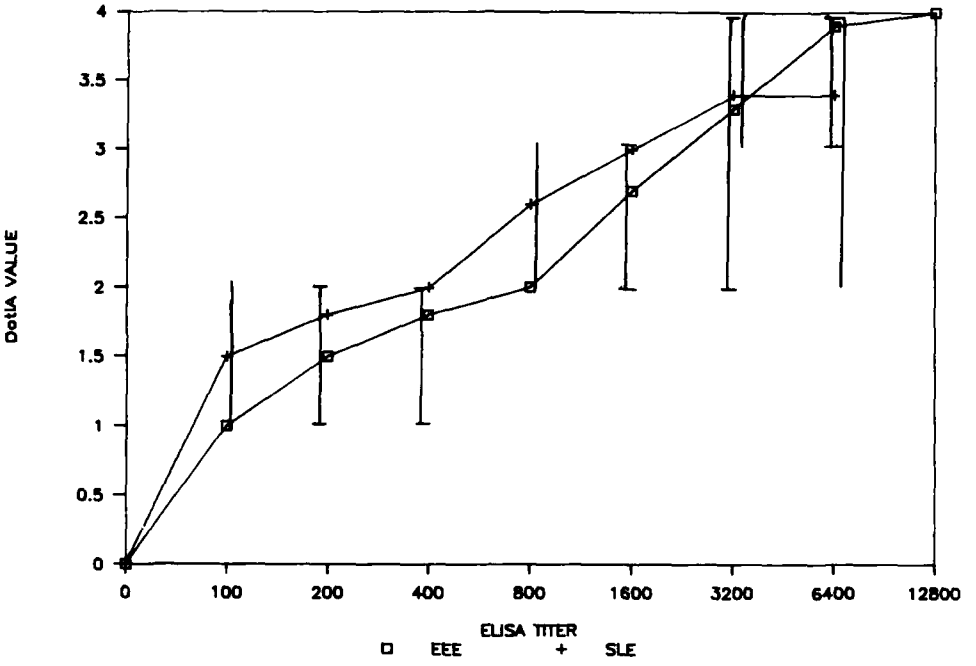

FIGURE 3. Composite DotIA values per ELISA titer for EEE- (□) and SLE-(+) inoculated chickens. Confidence intervals represent the range of DotIA values per ELISA titer. EEE confidence intervals are crossed. For EEE $r^2 = 0.62$, $P = 0.012$; for SLE $r^2 = 0.51$, $P = 0.046$.

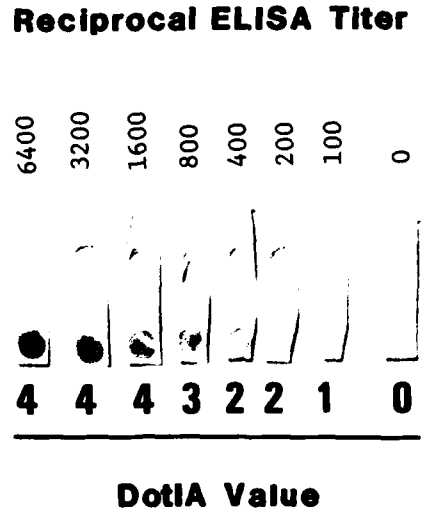

FIGURE 4. Multiple antigen DotIA assay strip for SLE/normal mouse brain/EEE (top to bottom). Each strip represents an assay of anti-EEE chicken sera of various EIA titers (serial two-fold from 6,400 to 100). Corresponding DotIA values are given.

to 800, 1 to 1,600, 2 to 3,200, and 2 to 6,400 by EIA. Two of 3 sera, titered to 800, had a DotIA value of +2; the third produced a DotIA value of +3. All samples titered to ≥1,600 by EIA had DotIA values of +4.

The DotIA protocol was shown to detect antibody to EEE virus at a reciprocal EIA dilution of 100 (Fig. 4). Sera with an EIA titer of 100 had a mean DotIA value of +1. EIA titers of 200–400 produced a DotIA value of +2, titers of 800 a value of +3, and sera titered at ≥1,600 had a DotIA value of +4.

High EIA titered (6,400) SLE virus antisera and EEE virus antisera were assayed in a heterologous DotIA to test for cross-reaction (Fig. 4); none was observed. HJ virus antisera (with EIA titers of 6,400) from experimentally infected birds were also assayed with EEE virus antigen strips. No cross-reactivity was observed.

DISCUSSION

We first attempted to perform DotIA on nitrocellulose (NC), as is done in other membrane

based immunoassays.[11,12] While a homologous test with mouse immune ascitic fluid (MIAF) worked well on NC, the chick sera assay did not. PVDF has a higher protein binding capacity and produces much lower backgrounds than NC.[13] Our data indicate that PVDF was superior to NC for this dot-immunoassay. NC was found unsuitable for some assay components due to either inefficient binding of target antigen or interference with antigen-antibody interactions.[17,18] PVDF is also less tractable than NC and thus preferable for field use.

The superior performance of PVDF in the DotIA assay is likely due to the strong binding of proteins to hydrophobic fibers[13–15] and the inert nature of the material of which the membrane is made. This results in higher signal and lower background. Suckling mouse brain antigen was found to yield the highest signal : noise ratio of the antigens tested; use of crude Vero cell culture as antigen resulted in higher backgrounds,[19] making it more difficult to discriminate IgG positive and negative specimens.

In the quantitative detection of antibody, the DotIA, in a format designed for speed, was less sensitive than conventional EIA. Serum samples, in the DotIA, were used at a working dilution of 1:10 whereas the beginning serum dilution in the EIA was 1:100. The minimum detectable titer of a serum sample in the EIA assay was 1:100. This was also the lowest titered (by EIA) serum sample considered positive in the DotIA test (at this test's working dilution of 1:10).

Sensitivity of the DotIA test in detecting seropositive animals was found to be equal to that of EIA. All samples of EEE virus- and SLE virus-infected chick sera that were positive by EIA were judged positive by DotIA. Correlation of negative samples for EEE and SLE chick sera was 95% (18/19) and 100% (35/35), respectively, as compared with EIA. The one EIA-negative EEE virus-inoculated chicken detected as positive by DotIA on day 3 had an EIA IgG titer of 800 on day 4.

DotIA color development increased logarithmically with an increase in EIA titer. This resulted in dark spots beginning at relatively low serum titers. High DotIA values (+3, +4) had corresponding EIA titers which were normally distributed around one point. Lower DotIA values (+1, +2), however, were found to correspond to a wider range of EIA titers than high DotIA values. The reason for these distributions was not determined but may be due to a greater influence of serum antibody avidity and affinity in the low titer samples while the effect of concentration may be a more predominant factor in high titer samples.

A dramatic rise in color development with titer could be an advantage to surveillance in the field. When assayed on a weekly or monthly basis, first detectable titers, in sentinel animals, are likely to be high. This was indicated by our sentinel flock data as well as data from Calisher et al.[6] where positive serum IgG EIA titers from sentinel chickens were >400 when assayed. Data from chickens experimentally infected with EEE virus and SLE virus indicate that serum antibody titers to these viruses rise to an EIA value of ≥800 within 1–2 days of the first detectable titer (J. G. Olson and T. W. Scott, personal communication).

As in the standard EIA assay, there was no cross-reactivity between EEE serum and SLE serum samples even at high concentrations of antibody. HJ virus is an alphavirus which might potentially cross-react in an EIA. HJ is also found in the same geographical area and is transmitted by mosquitoes.[16] Several samples of high titer anti-HJ serum from experimentally infected chickens were tested by DotIA. High titer (1:6,400) anti-HJ chicken sera was not judged as positive in this assay. A high titered stock of HJ virus was slightly cross-reactive in one EIA antigen detection system.[3]

The advantages of the DotIA test for field application are numerous. Using PVDF "dip-strips" allows for the screening of large numbers of samples. Antibodies to multiple antigens can be assayed on a single strip with a small amount of serum (perhaps peripheral blood in diluent). The test can be completed rapidly, producing results in ≤50 min. All steps are carried out at ambient temperature and require no use of instrumentation or mechanical devices. The test strips are stable for long periods of time. The assay is performed in Eppendorf tubes and conical tubes for washes. A screening for antibodies to six different agents could be performed in a thin Eppendorf tube with 250 μl of diluted serum and all components kept in a shirt pocket. Reacted PVDF strips are a permanent record of the results.

The PVDF DotIA test is not meant to replace EIA for the quantification of antigens and antibodies; its application is clearly in field use.

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